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(54) Title: DNA SEQUENCING METHOD

(57) Abstract

A method of sequencing a DNA molecule is disclosed. This method comprises the step of exposing the molecule to a mixture of dITP/dGTP in a sequencing elongation reaction whereby compression artifacts are reduced.

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DNA SEQUENCING METHOD

Background of the Invention

The development of dependable methods for sequence analysis of DNA (deoxyribonucleic acid) has lead to the success of recombinant DNA methodologies and the field of 5 genetic engineering. DNA sequencing is generally accomplished by the method of Sanger, et al. (Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977) and involves the *in vitro* enzymatic synthesis of single-stranded DNA starting from either a single- or double-stranded DNA template. In the original 10 embodiment of the protocol, a primer, usually a synthetic oligonucleotide 15 to 30 bases in length, is first annealed to its complementary sequence on the template of the single-stranded DNA to be sequenced. The 3'-end of this primer is extended by the Klenow fragment of *E. coli* DNA polymerase I in 15 the presence of 2'-deoxynucleoside 5'-triphosphates (dNTPs), one of which contains a radiolabel.

Four separate sequencing reactions are performed, each buffered reaction containing all four dNTPs (2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate 20 (dCTP), 2'-deoxyguanosine 5'-triphosphate (dGTP), and 2'-deoxythymidine 5'-triphosphate (dTTP), and a small amount of one specific 2',3'-dideoxynucleoside 5'-triphosphate chain-terminating agent (either ddATP, ddCTP, ddGTP, or ddTTP; or, in general, ddNTP).

25 By varying the ratio of the specific chain-terminating ddNTP to its dNTP analog in a particular reaction, the polymerase generates a population of fragments where a specific ddNTP is substituted at every possible position along the DNA template where the corresponding dNTP would have been 30 incorporated. Once the one-step labelling and termination step has been completed, an excess of all four dNTPs are added

-2-

to each reaction to "chase" all fragments not terminated by a specific ddNTP into higher molecular weight DNA.

The products of the four separate reactions are then fractionated and visualized in adjacent lanes on a high 5 resolution denaturing polyacrylamide gel system.

In 1987 Tabor and Richardson (Tabor, S. and C.C. Richardson, *Proc. Natl. Acad. Sci. USA* 84:4767-4771, 1987) described a modification of the basic Sanger protocol for use with T7 DNA polymerase which separated the labelling from the 10 termination step, or a two-step sequencing protocol. T7 DNA polymerase and a limiting amount of all four dNTPs, one of which was radiolabeled, were added to an annealed template and primer. During a short incubation step at a suboptimal polymerization temperature (e.g., room temperature) the 15 polymerase added one to several hundred dNTPs to the 3'-end of the primer, while also incorporating the radiolabeled dNTP in all of the extended fragments. At the end of the labelling step, the mixture was allocated equally into four separate termination reactions. Each termination reaction contained 20 nonlimiting concentrations of all four dNTPs and one specific ddNTP.

Following a second short incubation step at the optimal polymerization temperature for the DNA polymerase (DNAP) (e.g., 37°C), detection of the DNA fragments was as outlined 25 for the Sanger protocol. The final process in both of the radiolabeled sequencing protocols described above included reading the autoradiogram to generate an ordered DNA sequence and then manual entry of this sequence into a data base for subsequent manipulations.

30 In 1989 Murray (Murray, V., *Nucl. Acids Res.* 17:8889, 1989) described a novel method for sequence generation from DNA templates using ddNTP termination of the DNA fragments.

-3-

Murray applied a variation of the polymerase chain reaction (Mullis, K.B., et al., *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273, 1986; Saito, R.K., et al., *Science* 230:1350-1354, 1985) which has become known as "cycle sequencing". Cycle 5 sequencing has the advantage of using smaller amounts of template DNA than those sequencing methods described previously.

Since the mid-1980's commercially available DNA sequencing instruments have automated the gel electrophoresis, 10 data collection, sequence generation and data entry steps involved with the radiolabeled methods described above. In addition, particular automated instruments have taken advantage of certain dyes that emit photon energy when excited with a laser, eliminating the need to use radioactivity to 15 detect the separated DNA fragments. All of the instruments incorporate a high resolution polyacrylamide gel system for separation of the labelled DNA fragments. Each instrument also contains some form of detection system at a fixed point across the length of the gel near its bottom to detect the 20 fluorescent-labelled fragments as they migrate during electrophoresis.

There are at present commercially available automated instruments based upon the detection technologies of: (1) single fluorescent-labelled primers or dNTPs with the 25 sequencing reactions run and detected in separate lanes of a gel (Ansorge, W., et al., *Nucl. Acids Res.* 15:4593-4602, 1987), (2) primers labelled with four separate fluors (Smith, L., et al., *Nucl. Acids Res.* 13:2399-2412, 1985; Smith, L., et al., *Nature* 321:674-679, 1986) allowing all four reactions to 30 be run and detected in one lane on a gel, or (3) the same strategy as in (2), above, except with the substitution of

-4-

four different fluorescent-labelled ddNTPs for the labelled primers (Prober, J., et al., *Science* 238:336-341, 1987).

One problem encountered by all sequencing methodologies is sequence compression caused by DNA secondary structure 5 during electrophoresis. The relatively short DNA fragments produced in the sequencing reaction fold back upon themselves, forming tight interstrand loops and hairpin turns. Some of these structures have sufficient strength that they are not completely denatured by heating or electrophoresis through 7-8 10 M urea. The incompletely denatured fragments, which may result from either A:T or G:C base pairs, migrate faster through the gel matrix than surrounding fragments of similar length, causing fragments that should differ by one to a few nucleotides to comigrate and appear as overlapping peaks (see 15 Figure 1, arrow). Unambiguous sequence determination is impossible in areas where compression artifacts occur. One typical way to resolve the sequence in an area of a compression is to sequence the opposite DNA strand (Davies, R.W., *Gel Electrophoresis of Nucleic Acids, A Practical 20 Approach* IRL Press, pp. 148-149, 1985).

Due to the cost involved with opposite strand sequencing various other strategies have evolved to combat compressions. These alternate strategies include running the sequencing gels at elevated temperatures and/or substituting modified dNTPs 25 into the sequencing mixes. Two of these modified dNTPs usually are dATP and dGTP where the nitrogen molecule at position 7 on the base moiety has been changed to a carbon atom (c⁷dATP and c⁷dGTP, respectively). Both c⁷dATP and c⁷dGTP decrease by one the number of hydrogen bonds formed 30 during base pairing of these molecules with their respective dNTP counterparts. The overall effect is to lower the melting temperature between DNA strands where the c⁷dNTPs

-5-

are incorporated allowing more efficient strand denaturation, and thus decreasing the number of compressions affecting the sequence data. One drawback with this method is the high cost of these modified molecules.

5 Another tactic used in an attempt to lower the melting temperature between DNA strands is the use of 2'-deoxyinosine-5'-triphosphate (dITP) in place of dGTP. However, dITP is not an ideal substrate for DNA polymerases and the results can be strong stops throughout the data, i.e., where one peak should
10 appear from a fragment of specific size, three or more may be found co-migrating together. (See Barr P.J. et al., *Bio Techniques* 4 (5) :428-32, 1986.)

Needed in the art of DNA sequence analysis is an improved method that helps avoid DNA secondary structure artifacts
15 during electrophoresis.

Summary of the Invention

In general, the present invention relates to nucleic acid sequencing methods. In particular, the present invention relates to the use of a mixture of 2' deoxynucleoside
20 triphosphates for relief of sequencing gel compressions.

The present invention is an improved method of sequencing a DNA molecule comprising the step of exposing a template DNA molecule to a mixture of dITP and dGTP, or functional analogs, in the sequencing elongation reaction. By "DNA sequencing elongation reaction" we mean the elongation reaction occurring when a DNA template is exposed to a DNA polymerase, appropriate dNTPs, and specific chain terminators, such as ddNTPs. Preferably, the dI/dG mixture is between the molar ratios of 40:1 and 1:5. More preferably, the ratio is between
25 20:1 and 2:1. Most preferably, the ratio is 5:1.

- 6 -

In one embodiment of the present invention, the DNA polymerase used in the sequencing elongation reaction is a thermostable polymerase or a genetically engineered DNA polymerase. In another embodiment of the present invention, 5 the polymerase is an N-terminal-deleted polymerase or a mutated version thereof. In a preferred form of the present invention, the polymerase has the amino acid sequence of *Thermus aquaticus* DNA polymerase excluding the N-terminal 280 amino acid residues or the F667Y mutation thereof. In another 10 preferred form of the present invention, the polymerase is TAQUENASE, a commercially available modified sequencing enzyme (Δ 280, F667Y Tag DNA polymerase), or T7 DNAP.

In one preferred form of the invention, the sequencing reaction is part of a cycle-sequencing protocol. In another preferred form of the invention, the sequencing reaction is part of an automated sequencing protocol. In another preferred form of the invention, the sequencing reaction is coupled to a polymerase chain reaction.

It is an object of the present invention to prevent
20 compression artifacts generated in the gel during DNA
electrophoresis.

Other objects, features and advantages of the present invention will become apparent after one has reviewed the specification, claims and drawings.

25 Brief Description of the Drawings

Figure 1 is a chromatogram of nucleotides 87 - 121 of an automated sequencing reaction using t27 as the template with sequencing mixes containing dGTP with no dITP. The arrow denotes the compressed region.

- 7 -

Figure 2 is a chromatogram of nucleotides 87 - 121 of an automated sequencing reaction using t27 as the template with sequencing mixes containing c⁷dGTP. The arrow denotes the compressed region.

5 Figure 3 is a chromatogram of nucleotides 87 - 121 of an automated sequencing reaction using t27 as the template with sequencing mixes containing both c⁷dGTP and c⁷dATP.

10 Figure 4 is a chromatogram of nucleotides 87 - 121 of an automated sequencing reaction using t27 as the template with sequencing mixes containing both dITP and dGTP at a 5:1 molar ratio.

15 Figure 5 is a chromatogram of nucleotides 58 - 87 of an automated sequencing reaction using pCT41A as the template with sequencing mixes containing dGTP. The arrows denote the compressed regions.

Figure 6 is a chromatogram of the same DNA sequence as in Figure 5 of an automated sequencing reaction using pCT41A as the template with sequencing mixes containing c⁷dGTP. The arrow denotes the compressed region.

20 Figure 7 is a chromatogram of the same DNA sequence as in Figure 5 of an automated sequencing reaction using pCT41A as the template with sequencing mixes containing both c⁷dGTP AND c⁷dATP.

25 Figure 8 is a chromatogram of the same DNA sequence as in Figure 5 of an automated sequencing reaction using pCT41A as the template with sequencing mixes containing dITP and dGTP in a 5:1 molar ratio.

30 Figures 9A, 9B and 9C is a chromatogram of an automated sequencing reaction using M13 DNA as a template with sequencing reactions containing dITP (Figure 9A), a 1:5 molar ratio of dITP:dGTP (Figure 9B) and a 5:1 molar ratio of dITP:dGTP (Figure 9C)

Detailed Description of the Invention

The present invention discloses that a mixture of dGTP and dITP can release hairpin-like structures created during gel electrophoresis. (By "hairpin-like structures" we mean 5 a formation that occurs when the last base or bases in a sequencing-generated fragment hybridize to one or several internal bases within the same fragment.) We postulate that by mixing dI and dG the introduction of dITP in the sequencing template will be randomized, thereby the negative effects of 10 using only dITP will be minimized. As the DNAP enzyme is less efficient to introduce dITP than dGTP, a small amount of dGTP will be introduced even if there is an excess of dITP in the mixture, but some dITP will statistically be introduced. The presence of dITP will lower the denaturing temperature, and 15 thereby, the hairpin structures will be easier to dissolve.

The present invention is a method for determining a nucleotide sequence for a template nucleic acid by chain termination sequence methods. Typically, the invention comprises the steps of first annealing an oligonucleotide 20 primer to a template DNA molecule, and then adding deoxyadenine-5'-triphosphate (dATP); deoxythymine-5'-triphosphate (dTTP); a mixture of deoxyguanine-5'-triphosphate (dGTP) and deoxyinosine-5'-triphosphate (dITP), and deoxycytosine-5'-triphosphate (dCTP), or functional analogs 25 thereof, chain terminators and a DNA polymerase, wherein a complementary strand synthesis reaction occurs. One then analyzes the results of the complementary strand synthesis reaction so that a nucleotide sequence of the template nucleic acid may be obtained.

30 By "functional analog" we mean analogs of any the dNTPs, such as c^7 dATP and c^7 dGTP, that substitute for the dNTPs

- 9 -

typically used in the sequencing reaction and may be added for various reasons. For example, one might add alpha-thio-dNTPs in place of the standard dNTPs to resolve compressions.

By "chain terminators," we mean molecules that upon 5 incorporation will stop nucleic acid chain elongation. A preferable chain terminator is a dideoxynucleotide.

Therefore, the present invention relates to a novel formulation of dNTP mixes for use in the elongation reaction during DNA sequencing. The present invention involves 10 combining dITP and dGTP in sequencing mixes. The mixes help reduce gel artifacts known as compressions and allow unambiguous DNA sequence determination.

We envision that the method will be applicable to a number of DNA sequencing methods, such as cycle sequencing and 15 standard DNA sequencing, both automated and manual.

Of particular significance to the present invention is automated DNA sequencing. The Examples below describe a preferred method of automated DNA sequencing according to the AutoRead Sequencing Kit (Pharmacia Biotech Inc.). The method 20 of the present invention will be suitable for other automated sequencing reaction variations, however.

All methods of DNA sequencing suitable for the present invention have certain parameters in common. For example, when one prepares a DNA sequencing reaction, one prepares four 25 different mixes with a specific chain terminator, preferably a ddNTP such as ddATP, ddCTP, ddGTP, or ddTTP, and various amounts of all four dNTPs. This provides a mix in which the specific ddNTP may substitute with a certain statistical frequency for one of the dNTPs. In the method of the present 30 invention, one would substitute a particular molar ratio of dITP/dGTP of functional analogs thereof, for the dGTP typically found in the sequencing mixes.

-10-

A DNA template is exposed to one of the sequencing mixes and a DNA polymerase at buffer concentrations and pH appropriate for an elongation reaction to occur. Random incorporation of the dideoxynucleotide will provide a 5 staggered selection of elongation products, each ending at a nucleotide corresponding to the added dideoxynucleotide. Size fractionation of all four sequencing reactions will provide a method of determining the sequence of the template molecule. Typically, this size fractionation is via electrophoresis, 10 mass spectrometry or by the use of chromatographic methods. The sequence may be determined by reference to chromatograms (such as Figures 1 - 9) obtained from the size fractionated extension products by the use of electrophoresis. Each peak in these chromatograms represent the size of a DNA fragment 15 terminated with one of the specific ddNTPs. The original template sequence is revealed by combining the terminated size fractionated DNA fragments from the four (A, C, G and T) different elongation/termination reactions (such as in Figures 1-9).

20 When using the method of the present invention, one will notice a lessening of the compression artifacts normally observed during electrophoresis. The Examples below demonstrate the magnitude of the subsequent corrected separation. In general, the formerly compressed nucleotides 25 will now appear as normal, non-compressed nucleotides.

We have found, as disclosed in the Examples below, that molar ratios of dITP/dGTP of 40:1 to 1:5 are suitable for DNA polymerase in sequencing reactions. Preferably a ratio of 20:1 to 2:1 is used. Most preferably the ratio is 5:1. A 30 different molar ratio of dITP to dGTP might be optimum for different DNA polymerases. The present invention might also affect DNAPs differently with respect to sequence reading

length because of inherent enzyme variations in the capability to incorporate dITP.

Applicants envision that one would optimize the use and proper ratios of dITP and dGTP for other enzymes commonly

5 used in DNA sequencing reactions, such as Klenow fragment, T7 DNAP, Taq DNAP, SEQUENASE (Amersham), and modified DNAPs.

Preferably, thermostable polymerases such as *Thermus aquaticus* DNA polymerases (Taq) or other Taq-like polymerases would be useful. Particularly, end-terminal-deleted thermostable

10 polymerases or mutated versions thereof, especially TAQ DNA polymerases excluding the end terminal 280 amino acid residues or the F667Y mutations, such as KLENTAQ 1 (AB Peptides, Inc., St. Louis, MO USA) and TAQUENASE (Wayne Barnes, Washington University, St. Louis, MO USA), are envisioned to be useful.

15 Each DNAP may require differing concentrations of both dNTPs and ddNTPs for optimization of either sequence reading lengths or signal intensities, or both. Concentrations of dNTPs may also be varied depending upon the methodology, e.g., mixes used with radiolabelled dNTPs would contain at least one 20 dNTP at a substantially lower concentration than the other three for efficient label incorporation, while reactions for automated sequencing instruments may use higher concentrations of all dNTPs in the mixes to improve overall signal strength and read length.

25 One skilled in the art would recognize other variations or additions to the mixes that might be required for optimization of sequencing reactions using different DNAPs such as, but not limited to:

- (a) Using a different buffer system,
- 30 (b) Requiring a modified ddNTP (e.g., 3'amino-ddNTPs and T4 DNAP) for efficient fragment termination,

-12-

(c) Addition other components (e.g., thermostable DNA binding proteins, ammonium sulfate, labelled dNTPs or ddNTPs, Triton X-100).

(d) Adding either c⁷dATP or c⁷dGTP or both
5 together.

Optimization of dI/dG mixes for DNA polymerases

We envision that different DNA polymerases could have different optimization of the molar ratio of dI/dG.

Therefore, one would optimize the dI/dG sequencing mixes for
10 candidate DNA polymerases. One would typically examine an elongation reaction consisting of the candidate DNA polymerases and a DNA template and optimize several important parameters. These parameters would typically be:

(a) Change buffer (type and/or concentration) and pH.
15 (b) Add other components from the mixes, e.g., sodium chloride, c⁷dATP, potassium chloride, ammonium sulphate.
(c) Vary the dITP:dGTP ratio.

One would choose the optimum pH and reaction conditions for the DNA polymerase in question and then vary the dI/dG ratios accordingly and observe length of read, compression relief and lack or appearance of strong stops. Preferably, both compression relief and read length are optimized.

Examples

The following examples disclose the use of several DNA templates (M13, t27, pCT41A, and pCYPAC) and both thermostable and non-thermostable DNA polymerases to demonstrate the efficacy of the present invention in various types of sequencing reactions.

-13-

A. Materials and Methods

The following enzymes were used for these examples:

- (a) T7 DNA polymerase (Pharmacia Biotech Inc.),
- (b) SEQUENASE Version 2.0 (Amersham),
- 5 (c) KLENTAQ 1, a 280 amino acid N-terminal deletion of the DNAP from *Thermus aquaticus*, as disclosed in Barnes U.S. Patent No. 5,436,149,
- (d) TAQUENASE, a 280 amino acid deletion containing a F667Y mutation in the polymerase domain as disclosed 10 in Barnes, U.S. Patent No. 5,436,149 and Tabor, European Patent Application No. 0 655 506 A1.

Sequencing of double-stranded DNA was according to the AutoRead Sequencing Kit instruction manual (Pharmacia Biotech Inc.). Briefly, 5 μ g of template in a 10 μ L volume of 15 distilled water were denatured by the addition of 1.5 μ L of 1 N \pm 0.005 N sodium hydroxide (NaOH) and heating to 5 minutes at 65-70°C. The denatured DNA was then placed at 37°C where 1.5 μ L of 1 N \pm 0.005 N HCl, 2 μ L of AutoRead Kit Annealing Buffer, and 4-6 picomoles of sequencing primer containing a 20 labelled 5' end were added. After a further 10 minute incubation at 37°C, the above mixture was allowed to cool at room temperature for 5-10 minutes. Once cool, 2 μ L of T7 DNAP at a concentration of between 6-8 units/ μ L, 1 μ L of AutoRead Sequencing Kit Extension Buffer, and 3.5 μ L of dimethyl 25 sulfoxide were added. 5.4 μ L of the mixture were then immediately added to the AutoRead Sequencing Kit Sequencing Mixes (A, C, G, and T) which had been prewarmed at 37°C. Each reaction was allowed to continue at 37°C for 5 minutes when 6 μ L of AutoRead Sequencing Kit Stop Solution were added. The 30 reactions were kept cold until heat denatured and loaded on an automated DNA sequencing instrument.

-14-

Sequencing of single-stranded DNA was according to the AutoRead Sequencing Kit instruction manual (Pharmacia Biotech Inc.). Briefly, a 17 μ L mix was prepared containing 2 μ g of template, distilled water, 2 μ L of AutoRead Kit Annealing Buffer, and 2-4 picomoles of sequencing primer with a labelled 5' end. The mixture was heated at 65-70°C for 10 minutes and then cooled at room temperature for 10 minutes. 2 μ L of T7 DNAP at a concentration of between 2-4 units/ μ L and 1 μ L of AutoRead Sequencing Kit Extension Buffer were added and 4.5 μ L 5 of the mixture immediately aliquoted to 2.5 μ L of each 10 AutoRead Sequencing Kit Sequencing Mix (A, C, G, & T) which had been prewarmed at 37°C. The reactions were allowed to continue at 37°C for 5 minutes when 6 μ L of AutoRead Sequencing Kit Stop Solution were added. The reactions were 15 kept cold until heat denatured and loaded on an automated DNA sequencing instrument.

Sequencing mixes used in Sanger-type sequence analysis contain different substances depending upon which DNAP is used to generate the DNA fragments. Table 1, below, discloses 20 exemplary sequencing mixes used with both TAQUENASE DNA polymerase and T7 DNA Polymerase. T7 Sequencing Mixes (see Table 1, below) typically contain Tris-HCl, four dNTPs (A, C, G, and T), NaCl, and a specific ddNTP. Some thermostable DNAPs, such as Tag or TAQUENASE (see Table 1), omit the NaCl 25 from the mixes due to its inhibitory effect upon the enzyme.

Cycle sequencing of double-stranded DNA was according to the AutoCycle Sequencing Kit instruction manual (Pharmacia Biotech Inc.) for Tag and Klentaq 1 DNAPs. A modification of the Sequencing Mixes, as outlined in Table 1 and below, was 30 required for TAQUENASE. Briefly, an 18 μ L master mix was prepared containing 250 ng-1 μ g template DNA, buffer, 1-2 pmoles of labelled primer, 2.5 units of DNAP, 5 μ L of dNTP

-15-

Solution and 8 % dimethyl sulfoxide. 4 μ L of the Master Mix were then distributed to 2 μ L of each ddNTP solution, covered with mineral oil, and subjected to the following thermal cycling program:

5

TABLE 1

Taqenase DNA polymerase (5:1 dI:dG ratio)				
Component	A Mix	C Mix	G Mix	T Mix
Specific ddNTP	5 μ M	5 μ M	2.75 μ M	5 μ M
	1 mM	1 mM	1 mM	1 mM
	1mM	1 mM	1 mM	1 mM
	0.4 mM	0.4 mM	0.4 mM	0.4 mM
	1 mM	1 mM	1 mM	1 mM
	1.6 mM	1.6 mM	1.6 mM	1.6 mM
Tris-HCl, pH 9.5	50 mM	50 mM	50 mM	50 mM
T7 DNA Polymerase (5:1 dI:dG ratio)				
Component	A Mix	C Mix	G Mix	T Mix
Specific ddNTP	5 μ M	5 μ M	5 μ M	5 μ M
	1 mM	1 mM	1 mM	1 mM
	1mM	1 mM	1 mM	1 mM
	0.4 mM	0.4 mM	0.4 mM	0.4 mM
	1 mM	1 mM	1 mM	1 mM
	1.6 mM	1.6 mM	1.6 mM	1.6 mM
Tris-HCl, pH 9.5	50 mM	50 mM	50 mM	50 mM
NaCl	40 mM	40 mM	40 mM	40 mM

(a) A hot start at 95°C for two minutes,
 (b) 25-30 cycles of incubation at the following
 30 temperatures for the indicated times: 95°C for 36 seconds,
 followed by 50°C for 36 seconds, and then 72°C for 84 seconds,
 (c) One cycle holding the temperature at 4°C until
 electrophoresis.

The following templates were used for DNA Sequencing:
 35 t27 and pCT41A (templates chosen because of their known
 compression artifacts), M13 DNA, and plasmid pCYPAC.

-16-

Templates t27 and pCT41A are described below. M13 DNA is commonly available from many sources. Plasmid pCYPAC is a 130kb-construct.

5 B. Results

To demonstrate compression relief using this invention, two different templates containing three different known compression artifacts were subcloned using standard molecular biology techniques into double-stranded plasmid vector 10 (pT7T3 18U; Pharmacia Biotech Inc.). Each template was then sequenced with either T7 DNAP or a thermostable DNAP using modified sequencing mixes as described above for double-stranded templates.

(a) t27 is approximately a 400 base insert of the human 15 thymidine gene. (References for the original material are Flemington, E., et al., Gene 52:267-277, 1987 and Arcot, S.S., et al., J. Biol. Chem. 264:2343-2349, 1989.) The compression is 5'-GAG CCA TAG-3', about 100 bases into the sequence using the M13 universal primer (Pharmacia Biotech Inc.), and obscured 20 sequence data from the automated sequencing instrument is demonstrated by the chromatogram in Figure 1 (arrow).

Figures 1 - 4 are chromatograms generated during automated sequencing reactions of nucleotides 87 - 121 of template t27. Figure 1 is a chromatogram of a sequencing 25 reaction with sequencing mixes containing dGTP with no dITP. The arrow denotes the compressed region. Figure 2 is a chromatogram of a sequencing reaction with sequencing mixes containing c⁷dGTP. The arrow denotes the compressed region. Figure 3 is a chromatogram of a sequencing reaction with 30 sequencing mixes containing both c⁷dGTP and c⁷dATP. Figure 4 is a chromatogram of a sequencing reaction with sequencing mixes containing both dITP and dGTP at a 5:1 molar ratio.

-17-

Figures 2, 3, and 4 demonstrate compression relief using either c⁷dGTP, a combination of c⁷dATP and c⁷dGTP, or a 5:1 molar ratio of dITP to dGTP, respectively, in the sequencing mixes. While resolution of the compression improves when 5 c⁷dGTP is used, the best sequence data is seen using either the expensive mixture of c⁷dATP and c⁷dGTP or this invention.

(b) pCT41A is a 41 base synthetic oligonucleotide containing two compression sites cloned into PT7T3 181. (Reference for the original material is Tabor, S. and C.C. 10 Richardson, *Proc. Natl. Acad. Sci USA* 84:4767-4771, 1987.)

Figures 5-8 are chromatograms of nucleotides 58 - 87 of an automated sequencing reaction using pCT41A as the template. Figure 5 is a chromatogram of a sequencing reaction with sequencing mixes containing dGTP. The arrows denote the 15 compressed regions. Figure 6 is a chromatogram of a sequencing reaction with sequencing mixes containing c⁷dGTP. The arrow denotes the compressed region. Figure 7 is a chromatogram of a sequencing reaction with sequencing mixes containing both c⁷dGTP and c⁷dATP. Figure 8 is a chromatogram 20 of a sequencing reaction with sequencing mixes containing dITP and dGTP in a 5:1 molar ratio.

One of the compressions, approximately 65 bases into the sequence using the M13 universal primer, is resolved with c⁷dATP as demonstrated in Figures 5 and 7. The second 25 compression, approximately 75 bases into the sequence using the M13 universal primer, is resolved with c⁷dGTP as demonstrated in Figure 6. Surprisingly, the readability of the sequence is enhanced further with the resolution of both compressions, as demonstrated in Figure 8.

30 The dITP/dGTP sequencing mixes were tested using T7 DNA polymerase with an M13 template (see Figure 9A). Figure 9A, B and C is a chromatogram of an automated sequencing reaction

-18-

using M13 DNA as a template with sequencing reactions containing dITP (Figure 9A), a molar ratio of 1:5 dITP to dGTP (Figure 9B) and a 5:1 molar ratio of dITP to dGTP (Figure 9C).

The set of components examined in Figure 9A would not 5 give extended read lengths (e.g., greater than 800 bases) using an automated DNA sequencing instrument while continuing to resolve compression artifacts. Figure 9A demonstrates the inherent problems associated with T7 sequencing mixes substituting dITP for dGTP directly using the single-stranded 10 template M13mp18(+) strand; note the stops at approximately 78, 260 and 410 bases into the sequence. Figure 9B is the same template using a molar ratio of 1:5 dITP to dGTP in the sequencing mixes; note that the stops seen in Figure 9A are no longer present. The mixes disclosed in Table 1, demonstrated 15 in Figure 9C, also function to prevent the strong stops seen in Figure 9A.

Surprisingly, when the mix formulation from Table 1, above, was used with a thermostable DNAP, TAQUENASE, in a cycle sequencing reaction on pCYPAC template, sequencing read 20 lengths of greater than 1000 bases were achieved without compression artifacts.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Pharmacia Biotech, Inc.
- (B) STREET: 2202 North Bartlett Ave.
- (C) CITY: Milwaukee
- (D) STATE: Wisconsin
- (E) COUNTRY: United States of America
- (F) POSTAL CODE: 53202
- (G) TELEPHONE: (414) 456-4402
- (H) TELEFAX: (414) 266-8522

(ii) TITLE OF INVENTION: DNA SEQUENCING METHOD

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Quarles & Brady
- (B) STREET: 411 East Wisconsin Avenue
- (C) CITY: Milwaukee
- (D) STATE: WI
- (E) COUNTRY: U.S.A.
- (F) ZIP: 53202-4497

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Haas, George E.
- (B) REGISTRATION NUMBER: 27,642
- (C) REFERENCE/DOCKET NUMBER: 740211.90636

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (414) 277-5000
- (B) TELEFAX: (414) 271-3552

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-20-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGCCATAG

9

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TAATTTGGGC GTGCARCAGG TTTTGACTG TGACC

35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAATTTGGGC GTGAGCACAG TTTTGACTG TGACC

35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAATTTGGGC GTGAGCCACA GTTTTGACT GTGACC

36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-21-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAATTTGGGC GTGAGCCACA GTTTTGACT GTGACC

33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCTGGCGGCH GGGGAGCMHA CGAAAGGCCG

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTGGCGGAC TGGGGAGCTC CGCAAGGAGC CG

32

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTGGCGGAG CCTGGGAGCT CCGCCAAGAG CCG

33

-22-

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCTGGCGGAG CCTGGGAGCT CCGCCAAGAG CCG

33

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 506 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCATGCCTGC AGGTCGACTC TAGAGGATCC CCGGGTACCG AGCTCGAATT CGTAATCATG	60
GTCATAGCTG TTTCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC	120
CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC	180
GTTGCGCTCA CTGCCCCTT TCCAGTCGGG AACCTGTCG TGCCAGCTGC ATTAATGAAT	240
CGGCCAACGC GCGGGGAGAG GCGGTTGCG TATTGGCGC CAGGGTGGTT TTTCTTTCA	300
CCAGTGAGAC GGGCAACAGC TGATTGCCCT TCACCGCCTG GCCCTGAGAG AGTTGCAGCA	360
AGCGGTSCCA CGCTGGTTG CCCCAGCAGG CGAAAATCCT GTTGATGGT KGTTYCGMAA	420
TCGGCAAAAT CCCTTATAAA TCAAAAGAAT AGCCCGAGAT AGGGGTGAGT GTTGTCCAG	480
TTTGGAACAA GAGTCCACTA TTAAAG	506

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 505 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-23-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCATGCCTGC AGGTCGACTC TAGAGGATCC CCGGGTACCG AGCTCGAATT CGTAATCATG	60
GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC	120
CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC	180
GTTGCGCTCA CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT	240
CGGCCAACGC GCGGGGAGAG GCGGTTGCG TATTGGGCGC CAGGGTGGTT TTTCTTTCA	300
CCAGTGAGAC GGGCAACAGC TGATTGCCCT TCACCGCCTG GCCCTGAGAG AGTTGCAGCA	360
AGCGGTGCAC GCTGGTTGC CCCAGCAGGC GAAAATCCTG TTTGATGGTG GTTCCGAAAT	420
CGGCAAAATC CCTTATAAAAT CAAAAGAATA GCCCGAGATA GGGTTGAGTG TTGTTCCAGT	480
TTGGAACAAG AGTCCACTAT TAAAG	505

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 504 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCATGCCTGC AGGTCGACTC TAGAGGATCC CCGGGTACCG AGCTCGAATT CGTAATCATG	60
GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC	120
CGGAAGCATA AAGGTGTAAA GCCTGGGTG CCTAATGAGT GAGCTAACTC ACATTAATTG	180
CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA	240
TCGGCCAACG CGCGGGGAGA GGCGGTTGC GTATTGGGCG CCAGGGTGGT TTTCTTTTC	300
ACCAGTGAGA CGGGCAACAG CTGATTGCCCT TCACCGCCT GGCCTGAGA GAGTTGCAGC	360
AAGCGGTCCA CGCTGGTTG CCCCAGCAGG CGAAAATCCT GTTGATGGT GGTYCGAAAT	420
CGGCAAAATC CSTTATAAAAT CAAAAGAATA GCCCGAGATA GGGTTGAGTG TTGTTCCAGT	480
TTGGAACAAG AGTCACTATT AAAG	504

CLAIMS

We claim:

1. A method for determining a nucleotide sequence for a template nucleic acid by a chain termination sequence method comprising the steps of

- a) annealing an oligonucleotide primer to a template DNA molecule,
- 5 b) adding deoxyadenine-5'-triphosphate (dATP; deoxythymine-5'-triphosphate (dTTP); a mixture of deoxyguanine-5'-triphosphate (dGTP) and deoxyinosine-5'-triphosphate (dITP), and deoxycytosine-5'-triphosphate (dCTP), or functional analogs thereof, and chain terminators, and a DNA polymerase, wherein a complementary strand synthesis reaction occurs; and
- 10 c) analyzing the results of the complementary strand synthesis reaction so that a nucleotide sequence of the template nucleic acid may be obtained.

2. The method of claim 1, wherein the ratio of dITP:dGTP is between 40:1 and 1:5.

3. The method of claim 2, wherein the ratio of dITP:dGTP is between 20:1 and 2:1.

4. The method of claim 1, wherein the ratio of dITP:dGTP is 5:1.

5. The method of claim 1, wherein the mixture of dITP and dGTP comprises a mixture of c⁷dGTP and dITP.

6. The method of claim 1, wherein the polymerase is a thermostable DNA polymerase.

7. The method of claim 4, wherein the polymerase is an N-terminal deleted DNA polymerase or mutated version thereof.

8. The method according to claim 7, wherein the polymerase is selected from the group consisting of a polymerase having an amino acid sequence as that of *Thermus aquaticus* DNA polymerase excluding the N-terminal 280 amino acid residues and the F667Y mutation thereof.

9. The method of claim 1 wherein the polymerase is T7 DNA polymerase.

10. The method of claim 1, wherein the sequencing method is according to a cycle sequencing protocol.

11. The method of claim 1, wherein the sequencing method is according to an automated sequencing protocol.

12. The method of claim 1, wherein the template nucleic acid sequence is amplified by polymerase chain reaction.

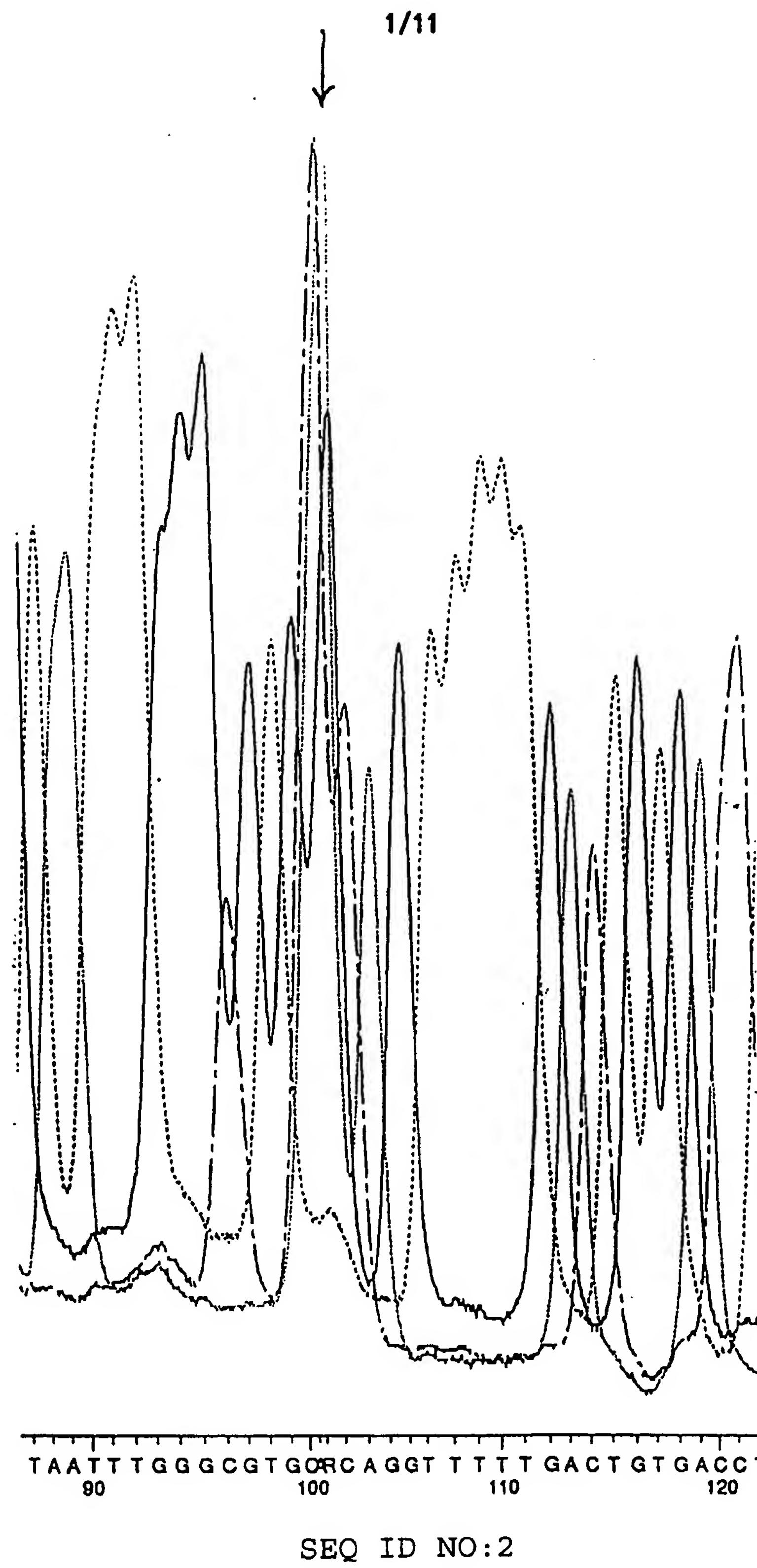
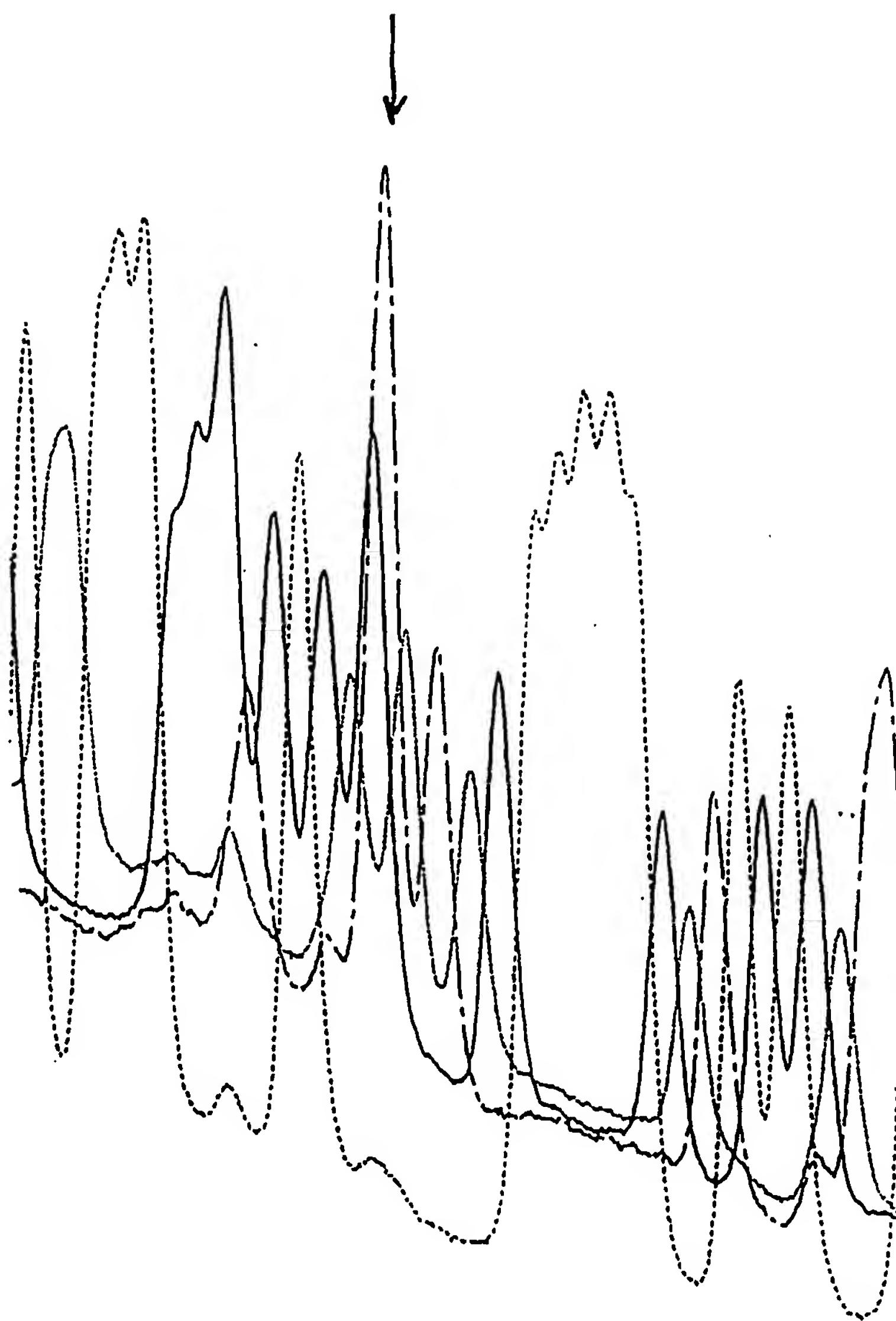


FIG. 1



SEQ ID NO: 3

FIG. 2

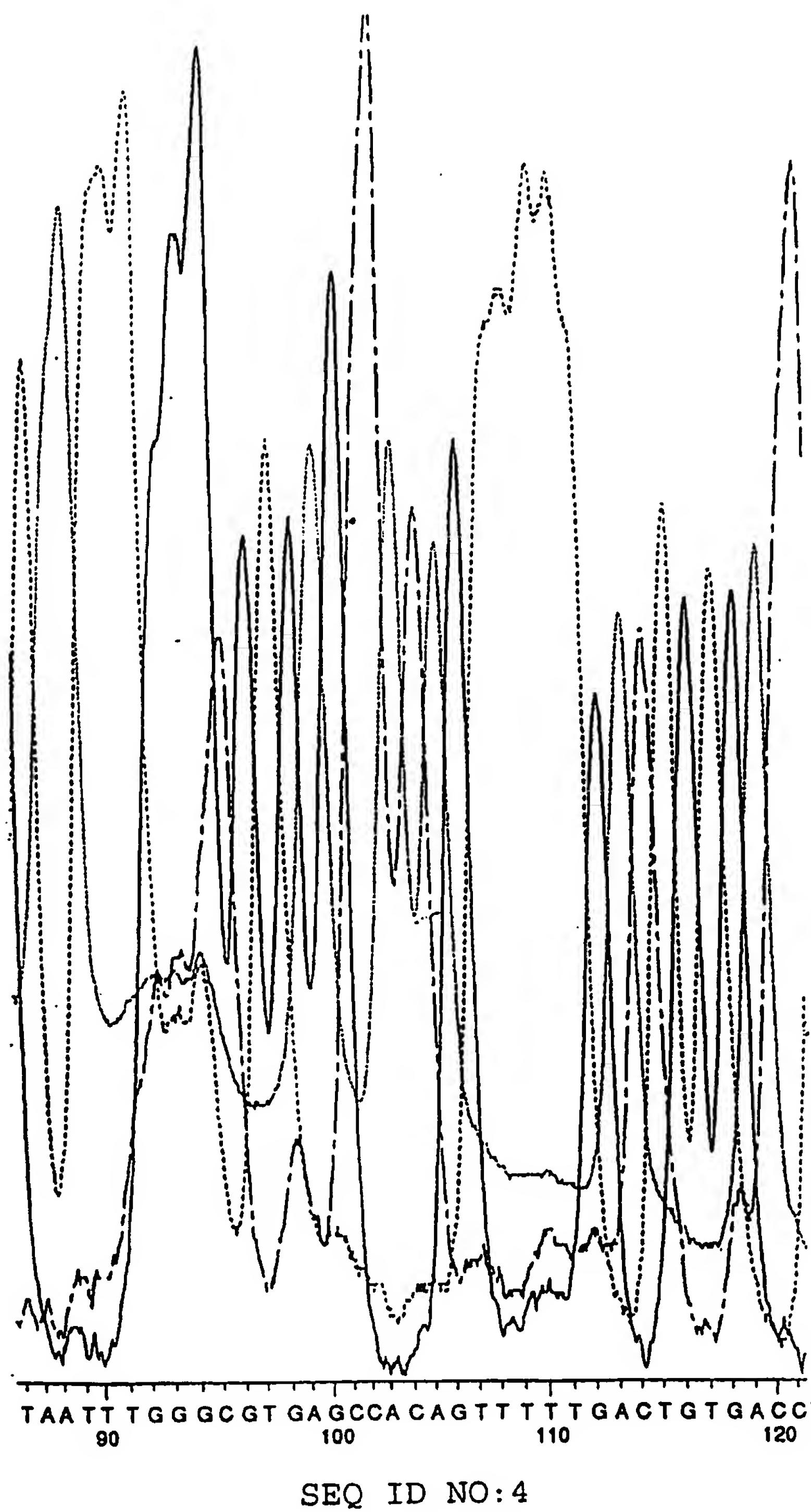
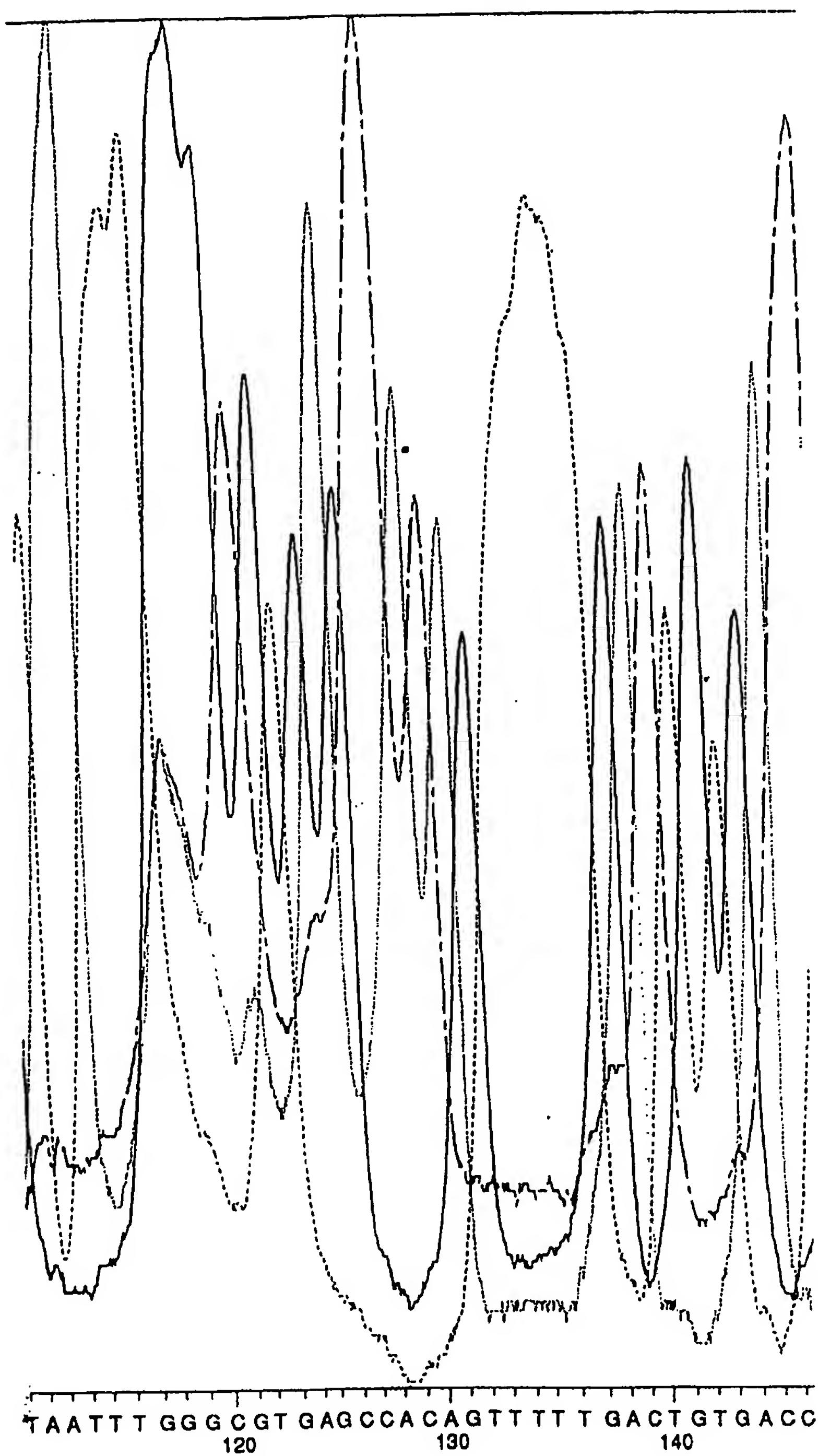


FIG. 3



SEQ ID NO:5

FIG. 4

5/11

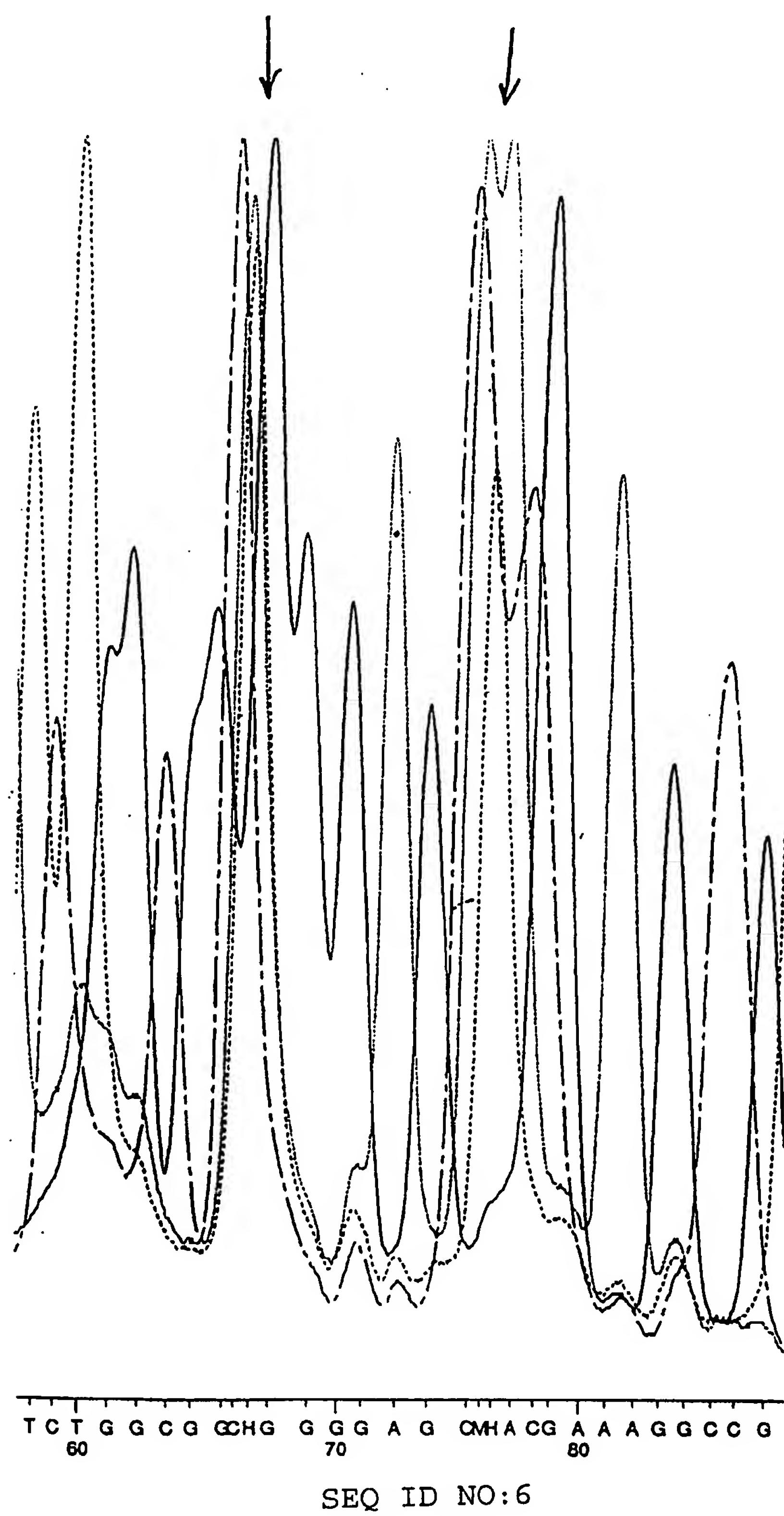
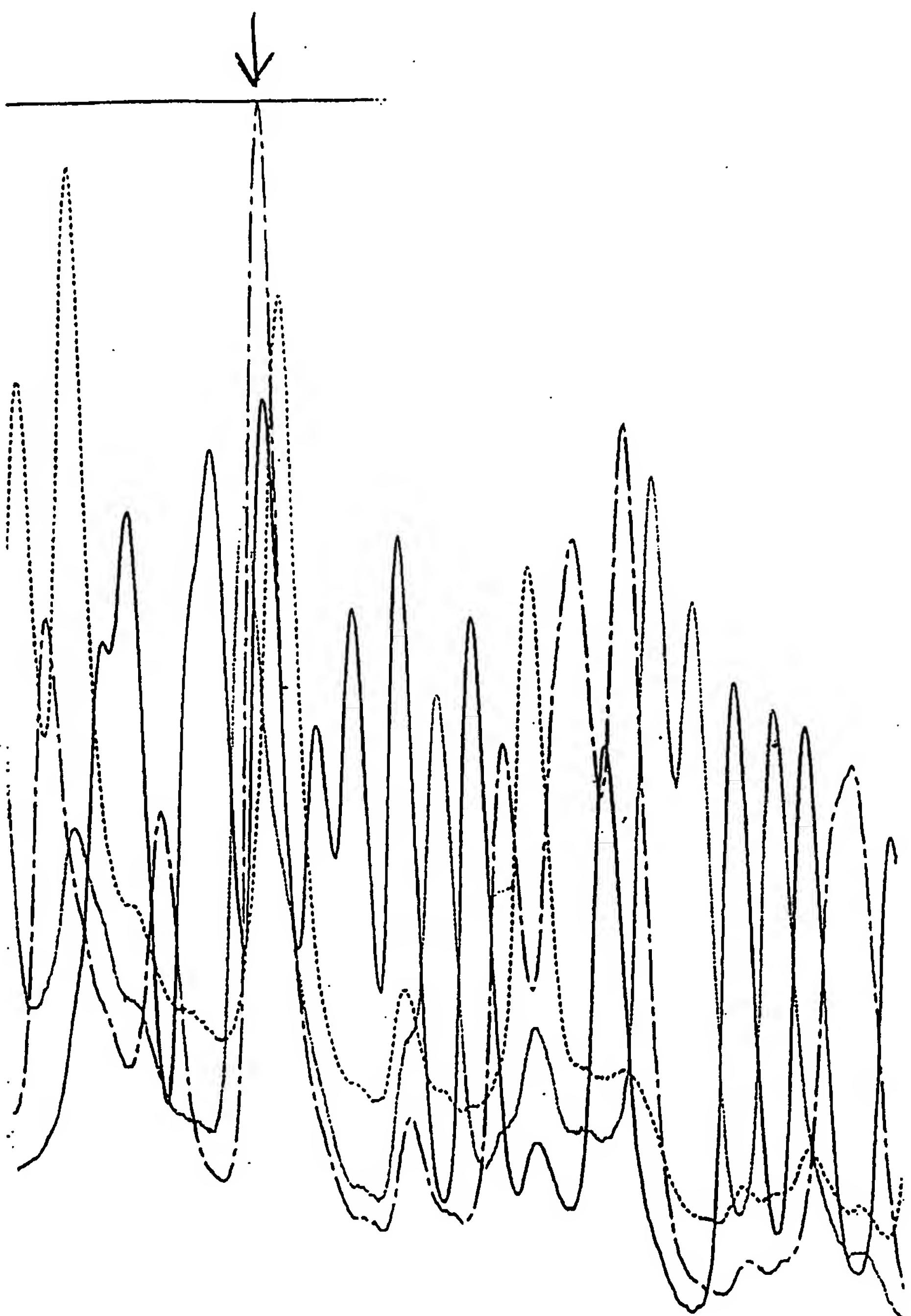
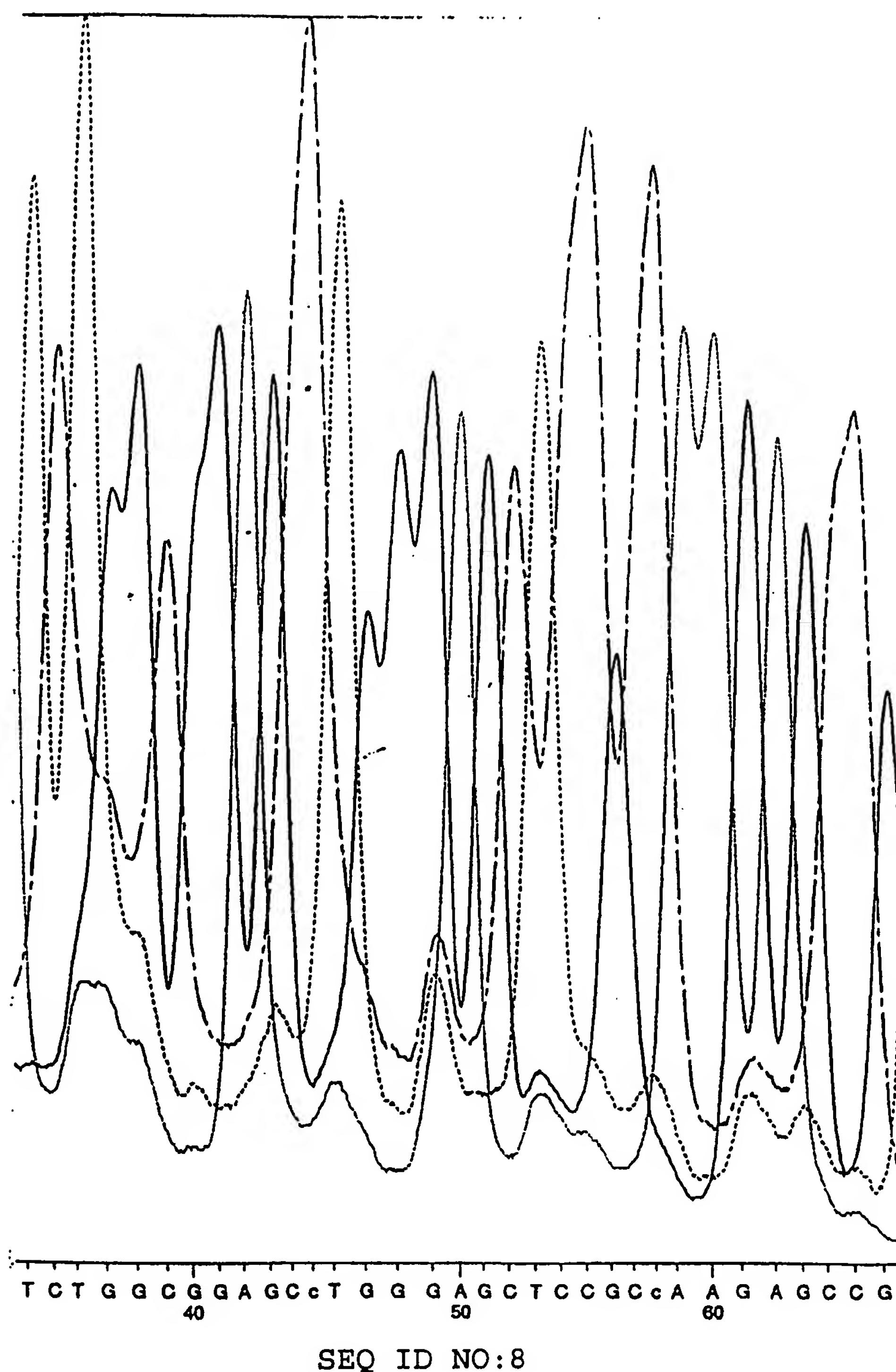


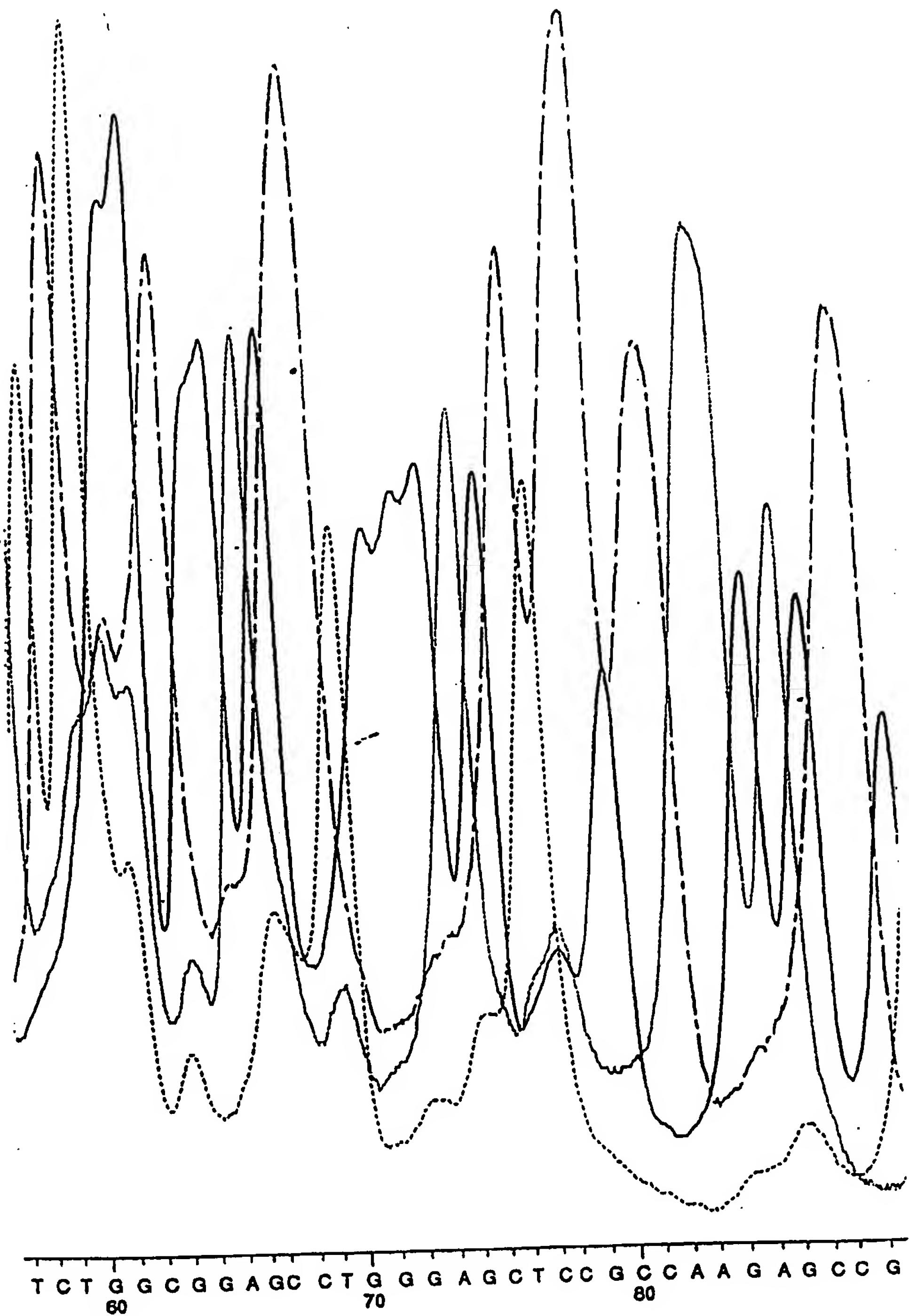
FIG. 5



SEQ ID NO: 7

FIG. 6

**FIG. 7**



SEQ ID NO: 9

FIG. 8

FIG. 9A

SEQ. ID NO. 10

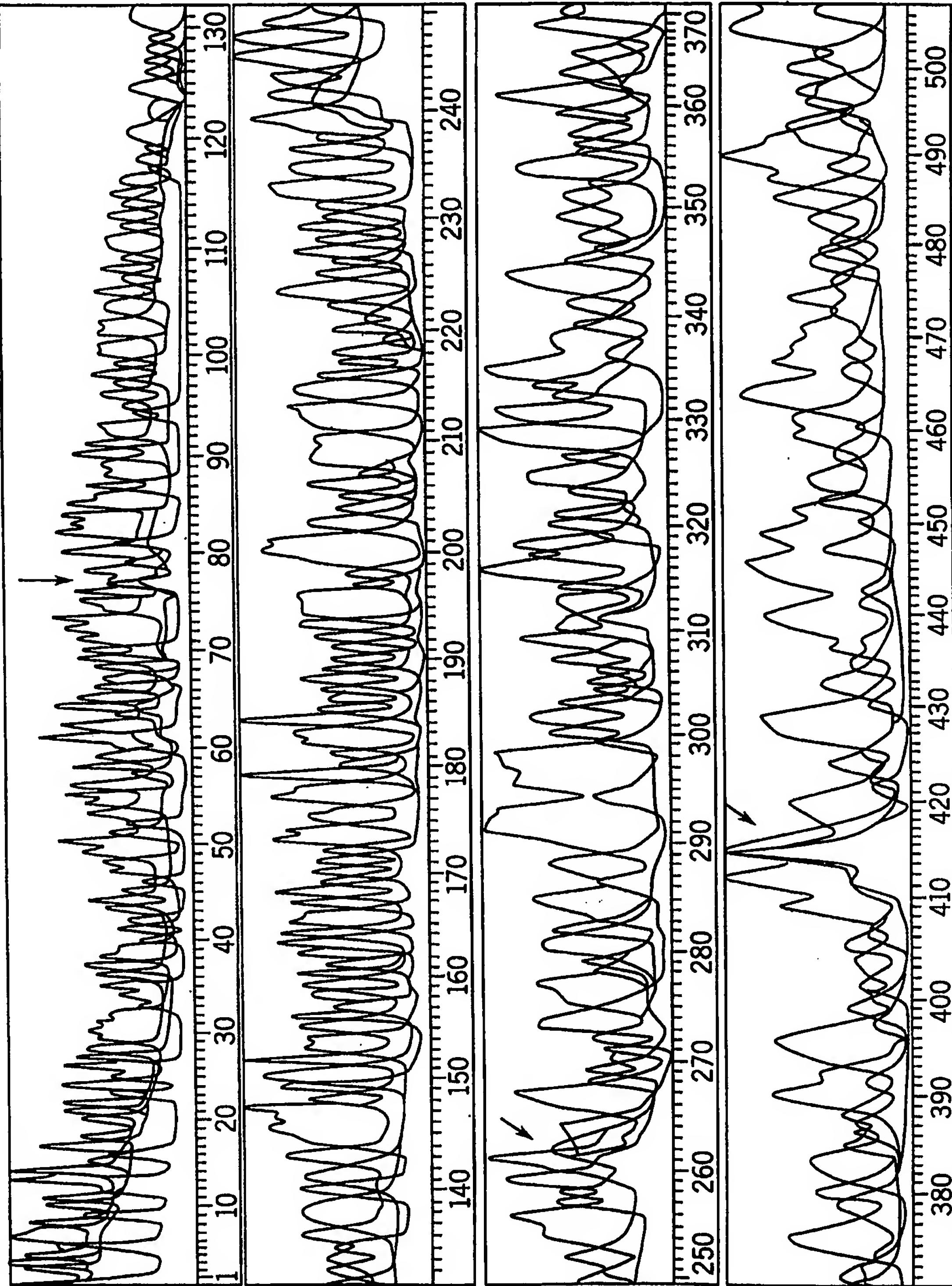
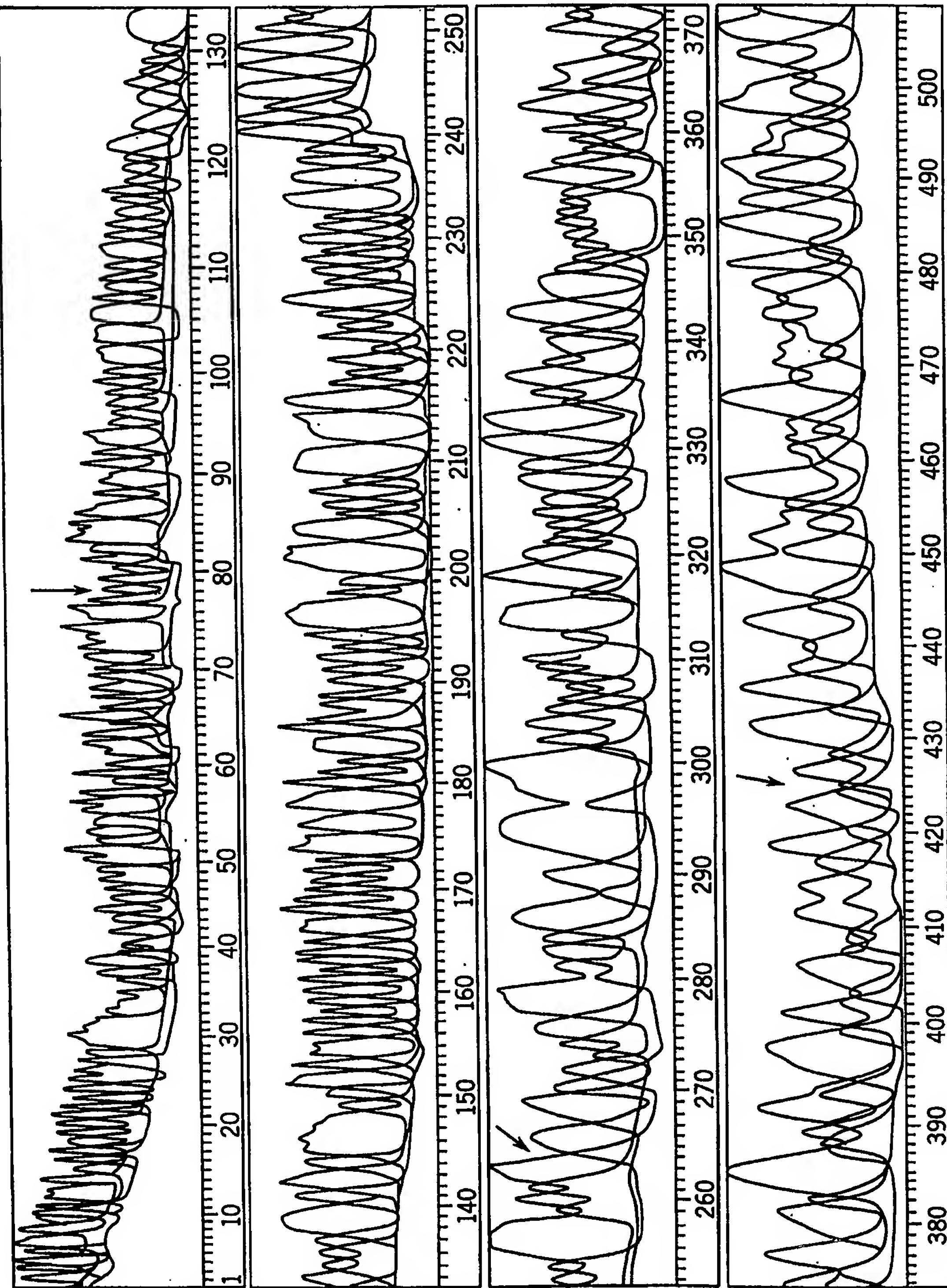
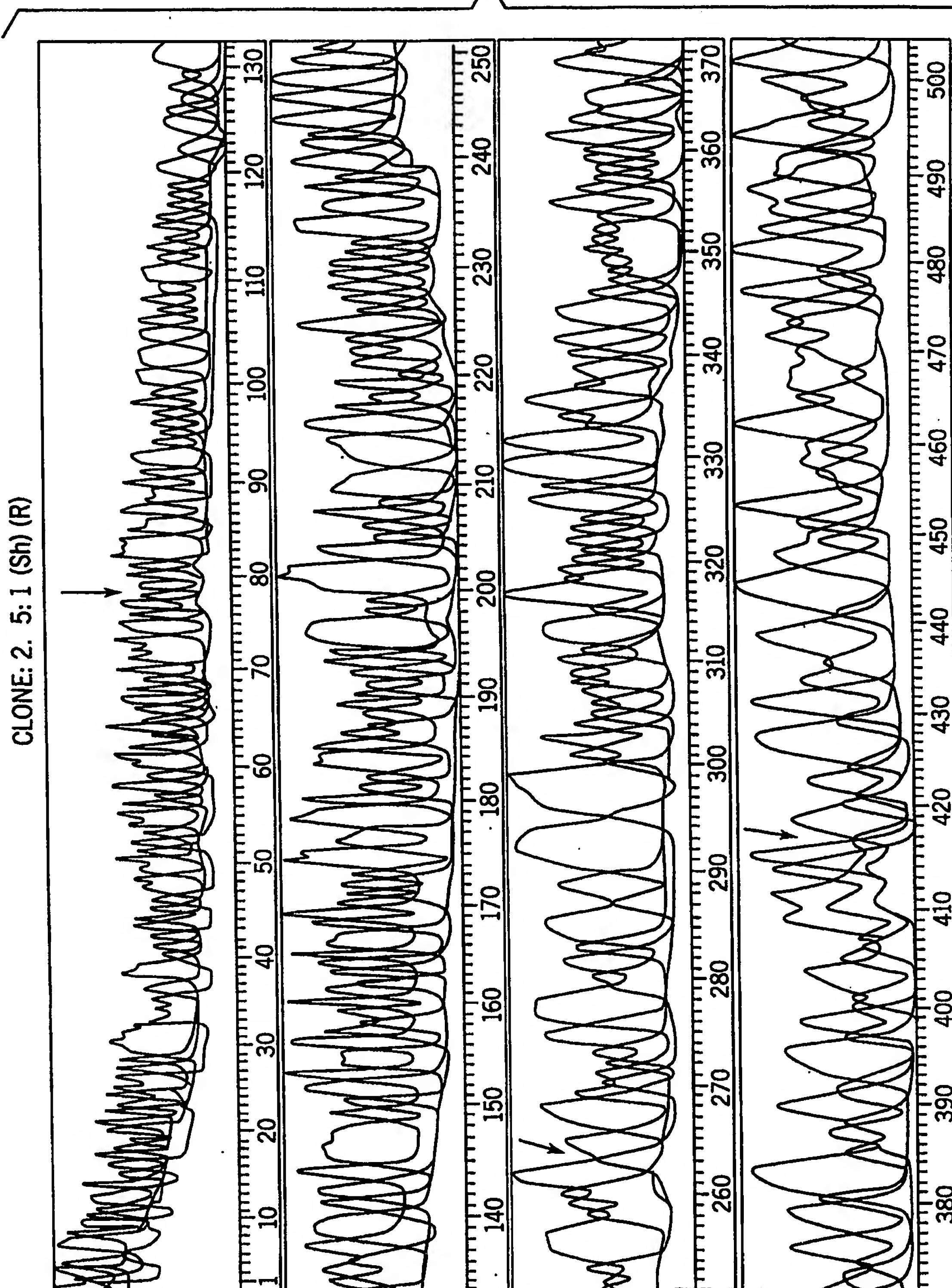
**SUBSTITUTE SWEET (RULE 28)**

FIG. 9B

SEQ. ID NO. 11
CLONE: 3. 1: 5 (Sh) (R)

SUBSTITUTE SHEET (RULE 26)

FIG. 9C



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/22902

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>INNIS M A ET AL: "DNA SEQUENCING WITH THERMUS AQUATICUS DNA POLYMERASE AND DIRECT SEQUENCING OF POLYMERASE CHAIN REACTION-AMPLIFIED DNA" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 85, no. 24, December 1988, pages 9436-9440, XP000611224 see the whole document</p> <p>---</p> <p>EP 0 629 706 A (AKZO NOBEL NV) 21 December 1994 see column 3, line 58 - column 4, line 11</p> <p>---</p> <p style="text-align: center;">-/-</p>	1-12
Y		1-12

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the International filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the International search

14 May 1998

Date of mailing of the International search report

27/05/1998

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Molina Galan, E

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/22902

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MILLS D R ET AL: "STRUCTURE-INDEPENDENT NUCLEOTIDE SEQUENCE ANALYSIS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 76, no. 5, May 1979, pages 2232-2235, XP002044087 see the whole document ----	1-12
A	EP 0 516 245 A (HARVARD COLLEGE) 2 December 1992 ----	
A	WO 90 03443 A (CETUS CORP) 5 April 1990 ----	
A	TABOR S ET AL: "DNA SEQUENCE ANALYSIS WITH A MODIFIED BACTERIOPHAGE T7 DNA POLYMERASE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 84, no. 14, July 1987, pages 4767-4771, XP000051702 cited in the application ----	
A	BARR ET AL.: "7-deaza-2'-deoxyguanosine-5'-triphosphate : enhanced resolution in M13 dideoxy sequencing" BIOTECHNIQUES, vol. 4, no. 5, 1986, pages 428-432, XP002065005 cited in the application -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/22902

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/22902

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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